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### Laminin $\alpha 4$ contributes to airway remodeling and inflammation in asthma

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1 **LAMININ  $\alpha$ 4 CONTRIBUTES TO AIRWAY REMODELING AND INFLAMMATION IN ASTHMA**

2

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23

24 **Running head:** Laminin  $\alpha$ 4 in ASM remodeling and inflammation.

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31

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34

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36   **Abstract**

37   Airway inflammation and remodeling are characteristic features of asthma, both contributing to  
38   airway hyperresponsiveness (AHR) and lung function limitation. Airway smooth muscle (ASM)  
39   accumulation and extracellular matrix deposition are characteristic features of airway remodeling,  
40   which may contribute to persistent AHR. Laminins containing the  $\alpha 2$  chain contribute to  
41   characteristics of ASM remodeling *in vitro* and AHR in animal models of asthma. The role of other  
42   laminin chains, including the laminin  $\alpha 4$  and  $\alpha 5$  chains, which contribute to leukocyte migration in  
43   other diseases, is currently unknown. The aim of the current study was to investigate the role of  
44   these laminin chains in ASM function and in AHR, remodeling and inflammation in asthma.  
45   Expression of both laminin  $\alpha 4$  and  $\alpha 5$  was observed in the human and mouse ASM bundle. *In vitro*,  
46   laminin  $\alpha 4$  was found to promote a pro-proliferative, pro-contractile and pro-fibrotic ASM cell  
47   phenotype. In line, treatment with laminin  $\alpha 4$  and  $\alpha 5$  function-blocking antibodies reduced allergen-  
48   induced increases in ASM mass in a mouse model of allergen-induced asthma. Moreover,  
49   eosinophilic inflammation was reduced by the laminin  $\alpha 4$  function-blocking antibody as well. Using  
50   airway biopsies from healthy subjects and asthmatic patients, we found inverse correlations  
51   between ASM  $\alpha 4$  chain expression and lung function and AHR, whereas eosinophil numbers  
52   correlated positively with expression of laminin  $\alpha 4$  in the ASM bundle. This study for the first time  
53   indicates a prominent role for laminin  $\alpha 4$  in ASM function and in inflammation, AHR and remodeling  
54   in asthma, whereas the role of laminin  $\alpha 5$  is more subtle.



## 55 Introduction

56 Asthma is a chronic airway disease, associated with airway inflammation and structural changes in  
57 the airway architecture, termed 'remodeling' (13). Both inflammation and remodeling may lead to  
58 airway hyperresponsiveness (AHR), defined as an exaggerated obstructive response to various non-  
59 specific stimuli (29). Airway remodeling includes increased airway smooth muscle (ASM) mass and  
60 contractility, and abnormal extracellular matrix (ECM) turnover resulting in an increased deposition  
61 (13). Studies on ECM modifications in asthma revealed an altered airway presence of several  
62 laminins (1, 2). Laminins are a group of heterotrimeric proteins comprised of five  $\alpha$ , three  $\beta$  and  
63 three  $\gamma$  chains (4). Together with collagen IV, nidogens and proteoglycans, laminins constitute the  
64 main functional components of basement membranes (BMs). In the healthy lung, laminin  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 5$ ,  
65  $\beta 1$ -3, and  $\gamma 1$ -2 are localized in the BMs beneath the airway epithelium, while laminin  $\alpha 4$ ,  $\beta 1$ -2 and  $\gamma 1$   
66 are present in ASM BMs (1, 8, 22, 25, 28, 42). Studies on the expression of the laminin chains in the  
67 airways of asthmatics are limited and focused on epithelial BMs. In these BMs, laminin  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 5$ ,  
68  $\beta 1$ -2 and  $\gamma 1$  were found to be increased (1, 2).

69 Increased ASM mass may be related to switching of the ASM cell between proliferative and  
70 contractile phenotypes (47). Exposure to proliferative stimuli induces a proliferative ASM phenotype,  
71 associated with increased synthetic function and reduced contractility, whereas removal of mitogens  
72 induces a contractile phenotype (47). In addition to their role as physical and mechanical support,  
73 laminins may also affect ASM phenotype switching. *In vitro*, laminin-111 (composed of laminin  $\alpha 1$ ,  
74  $\beta 1$  and  $\gamma 1$ ) inhibits ASM cell proliferation (9, 14, 18). Moreover, prolonged exposure of ASM cells to  
75 insulin and serum deprivation enhances laminin-211 ( $\alpha 2\beta 1\gamma 1$ ) expression, which also inhibits ASM  
76 proliferation (15, 40). In addition, laminin-111 prevents growth factor-induced reductions in ASM  
77 contractile protein expression and contractility, whereas laminin-211 increases contractile protein  
78 expression and contractility (9, 14, 15, 18, 40). Increased laminin-211 expression may also be  
79 important *in vivo*, as allergen-induced AHR was not observed in laminin  $\alpha 2$ -deficient mice (41).

80 Collectively, these findings suggest that laminins may importantly contribute to airway remodeling  
81 and AHR. However, the role of other laminin chains remains to be explored.

82 Airway inflammation closely relates to the development of variable and persistent AHR and is  
83 considered to contribute to the development, progression and maintenance of asthma (24).

84 Laminins are important regulators of immune cell migration (32). Laminin  $\alpha$ 4 promotes trans-  
85 endothelial migration of leukocytes, whereas laminin  $\alpha$ 5 restricts migration. Extravasation of  
86 leukocytes from blood vessels, in particular T-lymphocytes, but also monocytes and neutrophils,  
87 occurs predominantly at sites of low or absent laminin  $\alpha$ 5 expression (20, 33, 49).

88 The role of laminin  $\alpha$ 4 and  $\alpha$ 5 in abnormal ASM function in asthma has not been investigated yet.  
89 Therefore, the aim of the current study was to investigate the expression of laminin  $\alpha$ 4 and  $\alpha$ 5 in the  
90 airways and their role in airway smooth muscle function and airway remodeling and inflammation in  
91 asthma.

## 92    **Materials and methods**

93

94    Additional detail is provided in the online data supplement

95    (<https://figshare.com/s/e1f9577c4c54ed74fa04>).

96

### 97    *Human subjects and bronchial biopsies*

98    Airway wall biopsies from healthy subjects and patients with current asthma were obtained from  
99    two studies (5, 16, 35). Clinical and (immuno)histochemical parameters of the subjects in these  
100    studies have been reported (5, 16, 35). All subjects gave written informed consent. Studies were  
101    approved by the medical ethics committee of the University Medical Center Groningen. Biopsies  
102    used in the current study were selected on the presence of ASM, sections without ASM were  
103    excluded. Corresponding clinical characteristics are outlined in Table 1.

104

### 105    *Immunohistochemistry human biopsies*

106    Bronchial biopsies were cut into 3- $\mu$ m thick sections. Biopsy sections were stained with laminin  $\alpha$ 4  
107    and  $\alpha$ 5 antibodies, horse radish peroxidase-labeled secondary antibodies and diaminobenzidine,  
108    followed by a hematoxylin counterstain. Staining intensity was scored in triplicate by two  
109    independent observers in a blinded manner with scores ranging from 1-4 (Figure S1). The ASM,  
110    epithelium and endothelium were scored individually.

111

### 112    *Transplantation tissue and immunostaining*

113    Human tracheal sections from lung transplantation donors were used in this study (12). Tissue was  
114    collected according to the Research Code of the University Medical Center Groningen and national  
115    ethical and professional guidelines. Cryostat sections (4  $\mu$ m) were probed with pan-laminin, laminin  
116     $\alpha$ 4 or laminin  $\alpha$ 5 antibodies. Antibodies were a gift from dr. LM Sorokin of the University of  
117    Muenster, Germany. Antibodies were visualized by Alexa-488- or Cy3-labelled secondary antibodies.

Nuclei were labeled with Hoechst-33342. Sections were analyzed using an Olympus AX70 microscope and digital image capture system.

#### *Cell culture and lentiviral shRNA transduction*

Two human bronchial smooth muscle cell lines were used, immortalized by stable expression of human telomerase reverse transcriptase (17). Cells were used up to passage 30. Cells were transduced with  $3 \times 10^4$  infectious units lentiviral shRNA particles per well (2 ml) to knockdown laminin  $\alpha 4$  (sc-43147-V) or laminin  $\alpha 5$  (sc-43149-V) or with scrambled (control) shRNA lentiviral particles (sc-108080), according to the manufacturer's instructions. Preliminary results indicated this concentration of lentiviral particles to be maximally effective in reducing mRNA expression (data not shown). Stable clones were selected by growing transfected cells in medium containing puromycin.

#### *Real-time quantitative RT-PCR*

Real-time quantitative RT-PCR was performed using standard techniques. These data were analyzed using the comparative cycle threshold ( $C_T$ ) method. The amount of target gene was normalized to GAPDH. The specific primers used are listed in Supplemental Table S1.

#### *Cell proliferation assays*

Cell number was determined using both the AlamarBlue conversion assay and cell counting, using a hemocytometer (12). DNA synthesis was determined using the [ $^3\text{H}$ ]-thymidine incorporation assay (12).

#### *Animal provocations*

Inbred female BALB/c mice were obtained from Charles River. All animal care and experimental procedures complied with the animal protection and welfare guidelines, were approved by the Institutional Animal Care and Use Committee of the University of Groningen, The Netherlands.

Animal provocations were performed in two separate protocols, an acute protocol and a chronic protocol (Figure 3A). For both protocols, animals were sensitized on days 1, 14 and 21 by an intraperitoneal injection of ovalbumin (OVA) together with aluminum hydroxide in saline (7, 36). Animals were exposed to aerosolized 1% OVA or saline for 20 min, on day 28-30 (acute protocol) or days 26, 27, 33, 34, 40, 41, 47 and 48 (chronic protocol). In the acute protocol, animals were exposed to 5% OVA or saline on day 32 (7). To block laminin  $\alpha 4$  and  $\alpha 5$  function, 100  $\mu$ g of anti-laminin  $\alpha 4$  or anti-laminin  $\alpha 5$  IgG antibodies, respectively or control IgG antibodies were administered intravenously 1 day prior to the first aerosolized OVA exposure (45). In the chronic protocol, administration was repeated on day 39. Animals were sacrificed and lungs were harvested 6 hours (acute protocol) or 24 hours (chronic protocol) after the last OVA exposure. Time points were chosen so as to reflect maximum infiltration of eosinophils after allergen exposure (acute protocol) or to reflect remodeling and chronic inflammation (chronic protocol)(7, 21). ASM was visualized by sm- $\alpha$ -actin staining. Eosinophils were visualized by staining for cyanide resistant endogenous peroxidase activity. Airways within sections were digitally photographed and sm- $\alpha$ -actin staining and eosinophil numbers were quantified using Image J.

#### *Statistics*

Animal and cell data represents means $\pm$ SEM. For human experiments, data are presented as medians. Comparisons between two groups were made using Student's unpaired *t*-test (normally distributed data) or a Mann–Whitney *U*-test (non-parametric equivalent). Comparisons between three or more groups were performed using a one-way ANOVA, followed by Tukey's post-hoc test (normally distributed data) or Kruskal–Wallis *H*-test followed by Dunn's post-hoc test (non-normally distributed data). Correlations were calculated by non-parametric Spearman correlations. A value of *P*<0.05 was considered statistically significant. Analyses were performed with GraphPad Prism.

## Results

### *Expression of laminin $\alpha$ 4 and $\alpha$ 5 in human tissue*

To investigate the expression of laminin  $\alpha$ 4 and  $\alpha$ 5 in human airways, tracheal sections from lung transplant donors were stained with immunofluorescent antibodies. Staining with a pan-laminin antibody showed laminins to be present in the BMs of the epithelium, endothelium, airway and vascular smooth muscle, and submucosal glands (Figure 1). Laminin  $\alpha$ 4 was observed in the BMs of the airway and vascular smooth muscle and endothelium, but not the epithelium. Laminin  $\alpha$ 5 was observed in the BMs of the airway and vascular smooth muscle, epithelium, endothelium and submucosal glands.

### *Regulation of human ASM cell phenotype by laminin $\alpha$ 4 and $\alpha$ 5*

Given the important role of the ASM in AHR (13), we subsequently investigated the role of laminin  $\alpha$ 4 and  $\alpha$ 5 in ASM cell function. Laminin  $\alpha$ 4 mRNA is abundantly expressed by human ASM cells ( $\Delta C_T=12.1\pm 1.0$ ; Figure 2A), which is even higher than the abundantly expressed laminin  $\alpha$ 2 ( $\Delta C_T=13.4\pm 1.1$ ) (40). Laminin  $\alpha$ 5 mRNA was expressed much lower ( $\Delta C_T=17.5\pm 0.6$ ). Lentiviral knock-down of laminin  $\alpha$ 4 and laminin  $\alpha$ 5 significantly reduced expression of the respective laminin chains (Figure 2B-C). In addition, laminin  $\alpha$ 4 knock-down increased laminin  $\alpha$ 5 mRNA expression. No significant effects were observed on other laminin  $\alpha$  chains (Supplemental Figure S2). Laminin  $\alpha$ 4 knock-down significantly reduced baseline ASM cell proliferation, as indicated by cell number (Figure 2D), DNA synthesis and metabolic activity (Supplemental Figure S3). Silencing of laminin  $\alpha$ 4 reduced sm- $\alpha$ -actin and fibronectin mRNA expression, while no significant effects were observed on smooth muscle-myosin heavy chain (sm-MHC) and calponin expression (Figure 2E). Protein expression of sm- $\alpha$ -actin, calponin and fibronectin was also reduced in  $\alpha$ 4 deficient cells (Figure 2F-H). Silencing of laminin  $\alpha$ 5 significantly increased sm-MHC and calponin mRNA expression, however, this increase is

not carried through to the protein level. Collectively, these data indicate that laminin  $\alpha 4$  may play an important role in triggering a pro-fibrotic, pro-proliferative, and pro-contractile ASM phenotype.

#### *Laminin $\alpha 4$ and $\alpha 5$ regulate ASM remodeling in vivo.*

To explore the potential role of laminin  $\alpha 4$  and  $\alpha 5$  in ASM remodeling *in vivo*, we evaluated the effects of function-blocking antibodies in a mouse model of chronic allergic asthma (Figure 3A). This model is characterized by an allergen-induced inflammatory response, AHR and airway remodeling, including increased ASM mass (23). In this model, similar localization profiles were observed for laminin  $\alpha 4$  and  $\alpha 5$  in lung cryo-sections as for human tissue (Figure 3B). To investigate the role of both laminin chains in ASM remodeling, mice were treated with laminin  $\alpha 4$  or  $\alpha 5$  function-blocking antibodies. Allergen-induced ASM accumulation induced by repeated allergen challenge, as observed in control IgG-treated animals, was completely prevented by both laminin blocking-antibodies (Figure 3C). No effects were observed in saline-challenged animals. In addition, no effects were observed on ASM mass in the acute model (Figure S4). Collectively, these observations indicate that both laminin  $\alpha 4$  and  $\alpha 5$  are involved in allergen-induced ASM accumulation induced by repeated allergen challenge.

#### *Laminin $\alpha 4$ regulates eosinophil infiltration.*

Airway inflammation, particularly influx of eosinophilic granulocytes, closely relates to AHR in allergic asthma (24). As laminin  $\alpha 4$  and  $\alpha 5$  have been shown to be important in leukocyte migration (32), we next investigated their potential role in eosinophil infiltration in an acute and chronic challenge protocol (Figure 3A). In the acute model, the laminin  $\alpha 4$  function-blocking antibody significantly reduced both basal and allergen-induced increase in airway eosinophils (Figure 4A), whereas the laminin  $\alpha 5$  function-blocking antibody tended to decrease airway eosinophils ( $P=0.07$ ). In the chronic model, treatment with the  $\alpha 4$  antibody significantly reduced allergen-induced airway eosinophilia (Figure 4B). As in the acute model, no significant effect of the  $\alpha 5$  antibody was observed. No effects

were observed in saline-challenged animals. In both models, no significant effect of the antibodies on eosinophils surrounding the vasculature was observed either (data not shown). Collectively, these findings indicate that in addition to its role in ASM remodeling, laminin  $\alpha 4$  also contributes to leukocyte infiltration in a mouse model of asthma.

#### *ASM laminin $\alpha 4$ is associated with lung function, AHR and eosinophilia in asthma*

To investigate the potential role of laminin  $\alpha 4$  and  $\alpha 5$  expression in patients, staining intensity was scored in biopsies of healthy subjects and asthmatic patients (Figure S1). Staining patterns in airway biopsies (Figure 5A en 5B) were similar to those observed in tracheal sections (Figure 1). A small, but significant increase in endothelial laminin  $\alpha 4$  expression was observed in asthmatic patients compared to healthy controls (Supplemental Table S2). Surprisingly, a significant reduction in ASM laminin  $\alpha 4$  and laminin  $\alpha 5$  expression was observed in asthmatic patients. For laminin  $\alpha 4$ , this reduction appeared to be due to an interaction between smoking and asthma, as laminin  $\alpha 4$  expression was only significantly reduced in smoking asthmatics (Supplemental Figure S5). For laminin  $\alpha 5$ , the reduction observed in asthmatic patients was independent of smoking. No differences were observed in endothelial or epithelial laminin  $\alpha 5$  expression (Supplemental Table S2).

To investigate associations of laminin expression with clinical and biochemical parameters, expression was associated with previously published patient characteristics (5). Scores were grouped into low (score 1-2) and high (score 3-4). Interestingly, increased ASM laminin  $\alpha 4$  staining in the asthmatic patients was associated with reduced lung function (lower FEV<sub>1</sub> and lower FEV<sub>1</sub>/FVC) (Figure 5C-D), increased airway reactivity to adenosine monophosphate (AMP) (Figure 5E) and increased eosinophil numbers (Figure 5F). Similar associations were observed when smoking subjects were excluded from the analysis. Associations per individual staining score (1-4) are available in Supplemental Figure S6. No associations were observed for endothelial laminin  $\alpha 4$  staining (Supplemental Figure S7) or ASM laminin  $\alpha 5$  staining (data not shown). Higher endothelial



245 laminin  $\alpha$ 5 staining was associated with increased numbers of macrophages and reduced neutrophil  
246 numbers ( $P < 0.05$ , both; Supplemental Figure S8). No associations were observed with other  
247 parameters.

248 Previously, T-lymphocyte migration has been shown to be dependent on the laminin  $\alpha$ 4/ $\alpha$ 5  
249 ratio (49). In line with these observations, a laminin  $\alpha$ 4/ $\alpha$ 5 ratio of  $>1$  was associated with reduced  
250 lung function, increased AMP responsiveness and increased eosinophil numbers in asthma patients  
251 (Figure S9).

## Discussion

In the current study, we demonstrate for the first time that laminin  $\alpha 4$  may contribute importantly to the abnormal ASM function in asthma. *In vitro*, high expression of laminin  $\alpha 4$  by ASM cells was found and knock-down of this laminin reduced proliferation, contractile protein expression and ECM production, processes which are involved in airway remodeling, AHR and lung function decline in asthma. Accordingly, in asthmatic patients, ASM laminin  $\alpha 4$  expression was correlated with AHR, reduced lung function and increased eosinophil numbers and in an animal model *in vivo*, laminin  $\alpha 4$ -blocking antibodies reduced allergen-induced ASM remodeling and inflammation. Although no obvious effects were observed for laminin  $\alpha 5$  silencing *in vitro*, *in vivo* blockade of laminin  $\alpha 5$  prevented allergen-induced ASM increases.

Airway remodeling is a characteristic feature of chronic asthma and contributes to persistent AHR. Increased ECM deposition is an important characteristic of airway remodeling (13). Various ECM proteins, including collagens and fibronectin, are increased in the epithelial BM of asthmatic patients (6, 31). In addition, increased expression of several laminins, including laminin  $\alpha 5$ , have been reported (1, 2). In the current study no increase in epithelial laminin  $\alpha 5$  expression was observed. This may be explained by the parameter analyzed. In the current study, laminin staining intensity was scored, whereas in the previous study the thickness of the stained BMs was quantified (2). In the present study, intensity scoring was chosen as this method can also be used for other compartments, including the ASM. Increased ECM presence, including fibronectin and elastin, has also been shown in the ASM of asthmatic patients, which is related to airway function (3, 34). Another study showed no differences in the fractional area of ECM components in the ASM of asthmatic subjects (51). In that study, however, an inverse correlation was found between the fractional area of (pan-)laminin in the ASM bundle and FEV<sub>1</sub> reversibility (51). Remarkably, we show that both laminin  $\alpha 4$  and  $\alpha 5$  staining is reduced in the ASM of asthmatics. However, although laminin  $\alpha 4$  expression was reduced, there were significant associations between ASM laminin  $\alpha 4$  expression and lung function and AHR within the group of asthmatic patients. This paradoxical

observation might be related to an interaction between asthma and smoking. The mechanisms behind this interaction, however, are currently unknown and warrant further investigation.

The association between laminin  $\alpha$ 4 and lung function and AHR could be related to laminin  $\alpha$ 4-induced ASM cell phenotype changes. *In vitro* laminin  $\alpha$ 4 knock-down reduced ASM cell proliferation, contractile protein expression and fibronectin expression. These findings are in line with observations showing that ECM proteins are important regulators of ASM phenotype switching. Monomeric collagen I and fibronectin induce a proliferative phenotype, whereas laminin-111 and laminin-211 inhibit phenotype switching (9, 12, 14, 15, 18, 26). *In vivo*, treatment with a laminin  $\alpha$ 4-blocking antibody prevented allergen-induced ASM increase in a mouse model of asthma, supporting a role for laminin  $\alpha$ 4 in ASM abnormalities in asthma. The reduction in ASM mass may, however, also be (partly) indirect due to inhibition of eosinophil infiltration. Surprisingly, although only limited effects were observed for laminin  $\alpha$ 5 *in vitro*, *in vivo* blockade of this laminin fully prevented allergen-induced ASM accumulation. The mechanisms involved remain to be established.

Increased expression of contractile proteins has been observed in biopsies from asthmatic donors compared to non-asthmatic donors and may contribute to AHR (48). Expression of smooth muscle specific genes has been shown to be dependent on the binding of serum response factor (SRF) to the CArG [CC(A/T)<sub>6</sub>GG] box found in the promoters of contractile proteins, including sm-MHC and calponin (44). In line with an increased SRF binding, we found that expression of sm-MHC and calponin mRNA was increased in laminin  $\alpha$ 5 deficient cells. Increased expression of contractile proteins, including sm-MHC and calponin mRNA, has also been shown in tracheal smooth muscle tissue treated with antisense oligodeoxynucleotides directed against Integrin-linked kinase (ILK) (50). Reduced expression of ILK in these tissues resulted in an increased binding of SRF to the promoters of sm-MHC and calponin providing a potential link between integrins and contractile protein expression. Silencing of ILK resulted in an increased protein expression of sm-MHC, but not of calponin (50). In cultured ASM cells expression of sm-MHC is very low (17), therefore we were unable to quantify the effect laminin  $\alpha$ 5 knock-down on sm-MHC protein expression. Expression of

sm-MHC could thus be increased. Future studies using alternative culture systems should address whether sm-MHC protein expression requires laminin  $\alpha 5$ . Conversely, increased expression of calponin mRNA did not result in an increased protein expression, which is also in line with previous studies (50). Reasons for this discrepancy between mRNA and protein expression may be due to a number of factors, including differences in protein degradation, posttranslational mechanisms which regulate protein expression, differential expression of co-factors and/or amount of protein synthesis relative to basal protein expression (50).

Inflammation is a characteristic feature of asthma and contributes to both acute and persistent AHR (24). Acute AHR is transient and associated with episodic airway inflammation, whereas persistent AHR is associated with airway remodeling in response to recurrent airway inflammation (24). During extravasation, leukocytes cross the endothelial layer and penetrate the underlying BM. This step appears to be rate-limiting as leukocytes accumulate at the BM (38). Penetration of the BM depends on its composition (20, 33, 49). Laminin  $\alpha 4$  is ubiquitously localized in the endothelial BMs, while laminin  $\alpha 5$  distribution is patchy (49). Extravasation of T-lymphocytes, neutrophils and monocytes occurs predominantly at sites expressing no or low laminin  $\alpha 5$  (20, 43, 49), indicating that laminin  $\alpha 5$  may restrict, whereas laminin  $\alpha 4$  may promote extravasation. In the current study, we found that expression of endothelial laminin  $\alpha 4$  is increased in asthmatic patients, which could promote inflammatory cell infiltration (19). In line, inhibition of laminin  $\alpha 4$  using function-blocking antibodies prevented allergen-induced increases in eosinophil infiltration, both in the acute and chronic mouse model. In contrast to our expectations, no increase in inflammatory cell migration was observed in laminin  $\alpha 5$ -blocking antibody treated mice.

The relative expression of laminin  $\alpha 4$  in relation to laminin  $\alpha 5$  has been shown to regulate  $\alpha_6\beta_1$  integrin-dependent T cell migration across laminin  $\alpha 4$  matrices (49). Migration was maximal in the absence of laminin  $\alpha 5$  and decreased with increasing proportions of laminin  $\alpha 5$ , indicating that not only the absolute expression of laminin  $\alpha 4$ , but also the balance between laminin  $\alpha 4$  and laminin  $\alpha 5$  is important in these processes (49). In our studies, knock-down of laminin  $\alpha 4$  resulted in an

increased laminin  $\alpha 5$  mRNA expression in ASM cells. These findings are in line with previous studies in laminin  $\alpha 4$  knock-out mice showing an ubiquitous expression of laminin  $\alpha 5$  along the vessels, whereas expression of this laminin was patchy and lower in wild-type littermates (49). Although an increased laminin  $\alpha 5$  expression could contribute to the observed effects of the laminin  $\alpha 4$  knock-down in ASM cells, this is not very likely as we did not observe significant effects of laminin  $\alpha 5$  knock-down on proliferation or contractile protein in these cells. An effect of an altered laminin  $\alpha 4$  to laminin  $\alpha 5$  (laminin  $\alpha 4/\alpha 5$  ratio) could, which has previously been found to be relevant for lymphocyte migration across vascular laminins (49), on ASM function cannot be ruled out. In line, in our biopsy studies a laminin  $\alpha 4/\alpha 5$  ratio in the ASM bundle greater than 1 was associated with clinical characteristics in asthma.

Increased laminin  $\alpha 4$  expression in ASM was associated with increased numbers of eosinophils in asthmatic patients. Although regulation of laminin expression is poorly described, endothelial expression of laminin  $\alpha 4$  has been shown to be strongly upregulated by pro-inflammatory stimuli, such as lipopolysaccharide, interleukin- $1\beta$  and tumor necrosis factor- $\alpha$  (33), which may indicate that the increased ASM laminin  $\alpha 4$  expression is the result of inflammation.

Recently, a number of studies have shown that components of the laminin-integrin signaling axis may contribute to ASM abnormalities in asthma. Expression of CD151, a 4-transmembrane glycoprotein which associates with laminin-binding integrins, has been shown to be increased in the ASM of asthmatic patients (30). In these studies, CD151 was shown to associate with the laminin-binding  $\alpha 7\beta$  integrin and was required for G protein-coupled receptor-induced calcium mobilization in human ASM cells and AHR in a mouse model of asthma. Similarly, expression of integrin  $\alpha 7$  was shown to be increased in the ASM bundle of asthmatic patients (37). This integrin has previously been shown to be associated with a contractile ASM phenotype and knock-down of this integrin prevented the induction of a contractile phenotype (39). Moreover, this integrin was shown to be involved in ASM survival (41), suggesting that the laminin-integrin signaling axis may be involved in both increased ASM contractility as well as increased ASM mass, through reduced apoptosis. These

effects have thus far been attributed to the laminin  $\alpha 2$  chain, as induction of a contractile, hypoproliferative ASM phenotype was associated with increased expression of this laminin chain (15, 40). Moreover, allergen-induced AHR was not observed in laminin  $\alpha 2$  deficient mice (41). These effects of laminin  $\alpha 2$  in ASM cells are inhibited by the laminin  $\beta 1$  competing peptide Tyr-Ile-Gly-Ser-Arg (YIGSR) (15, 40, 41). In contrast to expectations from these in vitro studies, in vivo treatment with YIGSR attenuated the allergen-induced increase in ASM mass and enhanced ASM contractile protein expression and - contractility, both in control and in allergen-challenged animals (11). In the current study, we show that in addition to laminin  $\alpha 2$ , laminin  $\alpha 4$  also plays an important role in ASM abnormalities, providing a potential explanation for the contrasting observations with the YIGSR peptide in the in vivo model (11).

In conclusion, our results suggest that laminin  $\alpha 4$  is involved in airway remodeling and inflammation in asthma, which may contribute to lung function limitation and AHR. The role of laminin  $\alpha 5$  in these processes is less apparent and requires further investigation. On the basis of these results, laminin  $\alpha 4$  and/or its integrin ligand(s) may represent a novel target for the treatment of inflammation and airway remodeling in asthma.

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528 **Tables**

529

530 **Table 1** Clinical characteristics

	Control subjects	Asthmatic patients
Number	20	32
Gender (M/F)	11/9	12/20
Age (years) <sup>†</sup>	43 (19-65)	52 (19-71)
Current smoking, n (%)	9 (45)	6 (19)*
Atopy, n (%)	5 (25)	24 (67)***
β-agonist use, n (%)	0	18 (56)
ICS use, n (%)	0	15 (47)
FEV <sub>1</sub> (% pred) <sup>†</sup>	109 (83-132)	87 (34-134)***
FEV <sub>1</sub> /FVC (%) <sup>†</sup>	78 (71-88)	67 (40-86)***
Reversibility (%) <sup>†</sup>	2.7 (-7.4-8.2)	8.7 (1.3-38.4)***
PC <sub>20</sub> AMP (mg/ml) <sup>†</sup>	>320 (231.1 to >320)	8.7 (0.01 to >320)***

531 <sup>†</sup>Data are presented as median (range). Atopy is defined as the ratio of the concentration of specific

532 IgE's in patient serum relative to the concentration of specific IgE's in control serum >1 (5). \**P*<0.05,

533 \*\*\**P*<0.001 versus control subjects.

## Figure legends

**Figure 1** *Laminin  $\alpha 4$  and  $\alpha 5$  are expressed in human airways.* Stainings were performed on tracheal sections were obtained from one lung transplantation donor. (A) Localization of pan-laminin (green) in the basement membranes of the endothelium (Endo), epithelium (Epi), submucosal glands (SG) and airway smooth muscle (ASM). (B) Localization of laminin  $\alpha 4$  (green) in the basement membrane of the endothelium and airway smooth muscle. (C) Expression of laminin  $\alpha 5$  (red) in the basement membrane of the epithelium, endothelium, submucosal glands and airway smooth muscle. Pictures were taken at a 400 $\times$  magnification. Blue: nuclei.

**Figure 2** *Laminin  $\alpha 4$  is involved in the induction of a pro-fibrotic, pro-proliferative, and pro-contractile ASM phenotype in vitro.* (A) Laminin mRNA expression by human ASM cells. (B) Laminin  $\alpha 4$  mRNA expression is reduced by lentiviral shRNA directed against this laminin. No effects were observed for lentiviral shRNA directed against laminin  $\alpha 5$ . (C) Laminin  $\alpha 5$  mRNA expression is reduced by lentiviral shRNA directed against this laminin. Lentiviral shRNA directed against laminin  $\alpha 4$  increased laminin  $\alpha 5$  mRNA expression. (D) Cell number is reduced in laminin  $\alpha 4$  deficient cells. (E) Regulation of sm-MHC, calponin, sm- $\alpha$ -actin and fibronectin mRNA expression in laminin  $\alpha 4$  and  $\alpha 5$  deficient cells. (F-H) Protein expression of sm- $\alpha$ -actin, calponin and fibronectin is reduced in laminin  $\alpha 4$  deficient cells. Data represent means $\pm$ SEM of 4-12 experiments. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 compared to scrambled shRNA transfected cells.

**Figure 3** *Laminin  $\alpha 4$  and  $\alpha 5$  are involved in airway smooth muscle accumulation in vivo.* (A) Experimental animal procedures. Female BALB/c were sensitized to ovalbumin (OVA) on Days 1, 14, and 21. For the acute protocol, mice were challenged with saline or 1% OVA aerosols for 20 minutes on days 28-30. On day 32, animals were exposed to saline or 5% OVA and sacrificed 6 hours thereafter. For the chronic protocol, animals were exposed to saline or 1% OVA aerosols twice weekly from days 26 to 48. Mice were sacrificed 24 hours after the last challenge. Laminin  $\alpha 4$  or  $\alpha 5$

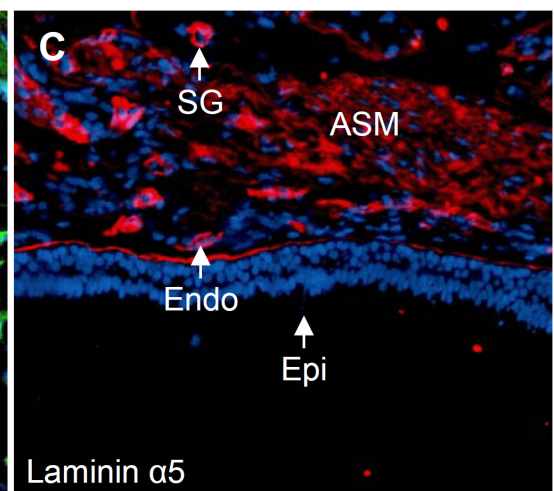
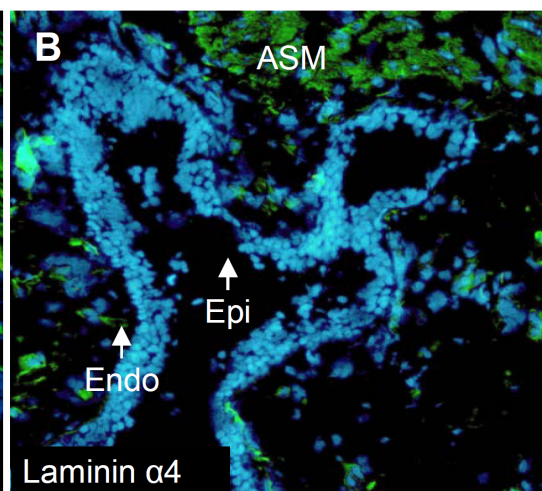
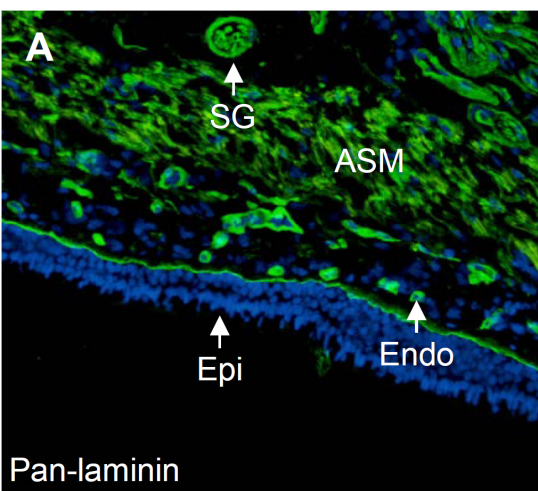
function-blocking antibodies or control IgG antibodies were administered on day 27 (acute protocol) or days 25 and 39 (chronic protocol). (B) Representative photographs of laminin  $\alpha 4$  and  $\alpha 5$  staining in mouse lung tissue after repeated ovalbumin challenge. Localization of laminin  $\alpha 4$  (green) in the basement membrane (BM) of the alveoli (AV) and airway smooth muscle (ASM). Expression of laminin  $\alpha 5$  (red) in the BM of the alveoli, epithelium (Epi) and ASM. Pictures were taken at 400 $\times$  magnification. AW: airway, V: vessel. Blue: nuclei. (C) Treatment with laminin function-blocking antibodies prevented allergen-induced ASM accumulation in the chronic model.  $^{\#}P<0.05$ ,  $^{\#\#}P<0.01$  compared with IgG-treated, ovalbumin-challenged controls. Data represent means $\pm$ SEM of 3-6 animals.

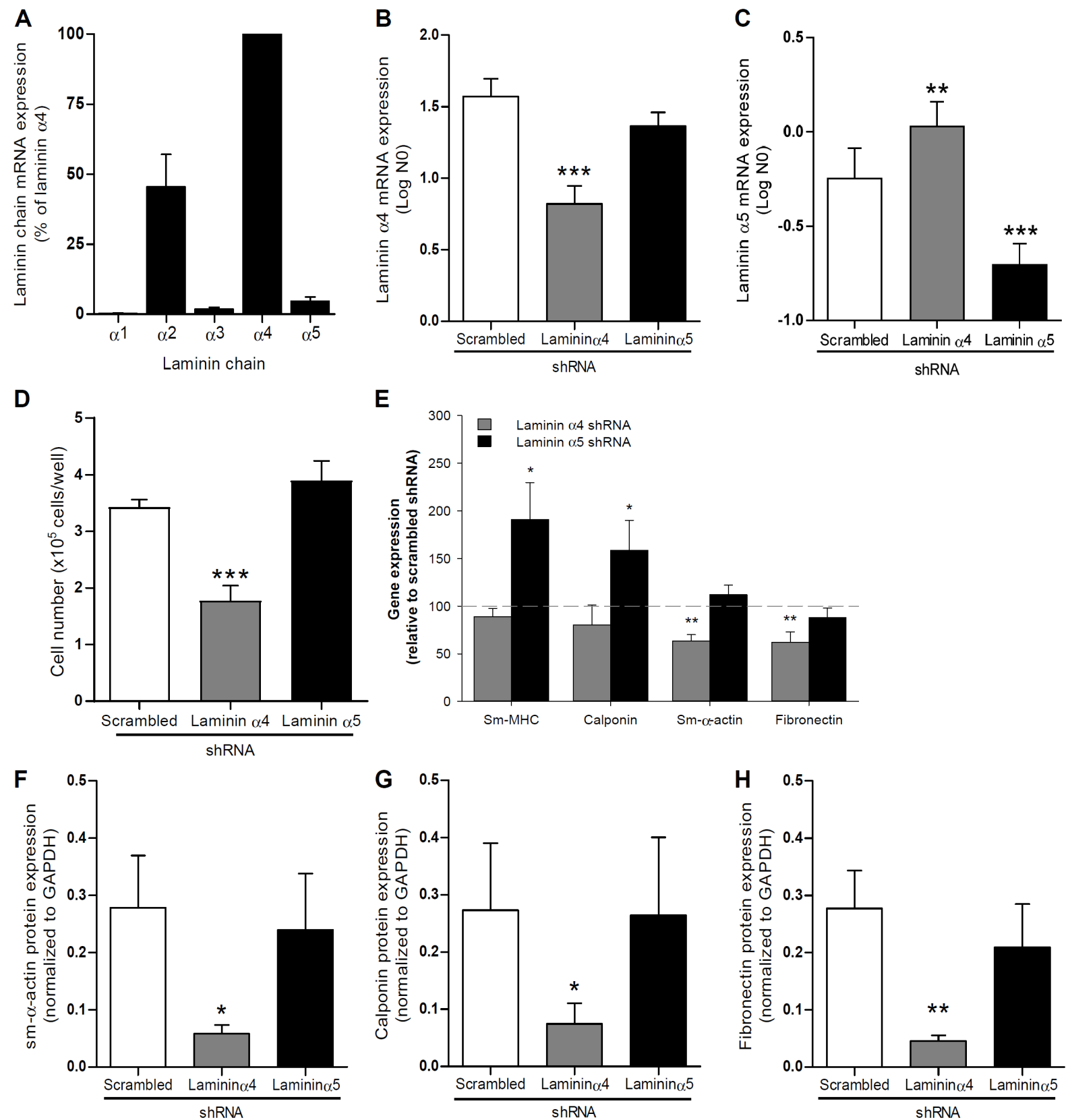
**Figure 4** *Basal airway eosinophil numbers and allergen-induced increases in eosinophils are inhibited by laminin  $\alpha 4$  blocking antibodies.* (A) Effects of laminin function-blocking antibodies on acute allergen-induced airway infiltration of eosinophils. (B) Effects of laminin function-blocking antibodies on chronic allergen-induced airway infiltration of eosinophils.  $^*P<0.05$  compared to with IgG-treated, saline-challenged controls.  $^{\#}P<0.05$  compared with IgG-treated, ovalbumin-challenged controls. Data represent means $\pm$ SEM of 3-6 animals. BM: basement membrane.

**Figure 5** *Laminin  $\alpha 4$  scoring is associated with clinical characteristics of asthmatic patients.* (A,B) Representative photographs of staining for (A) laminin  $\alpha 4$  and (B) laminin  $\alpha 5$  in ASM, epithelium and endothelium in biopsy sections. (C,D) High (score 3-4) laminin  $\alpha 4$  staining is associated with reduced lung function of asthmatic patients expressed as both (C) FEV<sub>1</sub> %predicted and (D) FEV<sub>1</sub>/FVC %predicted. (E) High (score 3-4) laminin  $\alpha 4$  staining is associated with increased airway reactivity of asthmatic patients to adenosine monophosphate (AMP). (F) High (score 3-4) laminin  $\alpha 4$  staining is associated with increased numbers of EPX-positive eosinophils in the airway biopsies of asthmatic patients. Results from 20 control subjects and 31 asthmatic patients are shown

585 in Figs C-F.  $**P<0.01$ ,  $***P<0.001$ . Examples of staining intensity scores 1-4 are shown in  
586 supplemental Figure S1.  
587

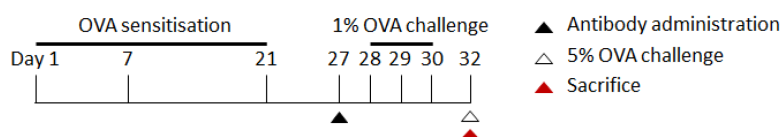




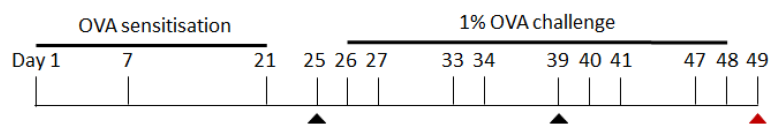


**A**

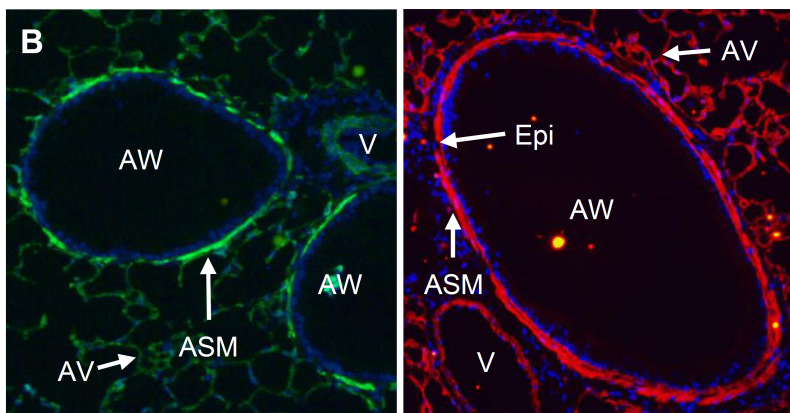
**Acute protocol**



**Chronic protocol**



**B**

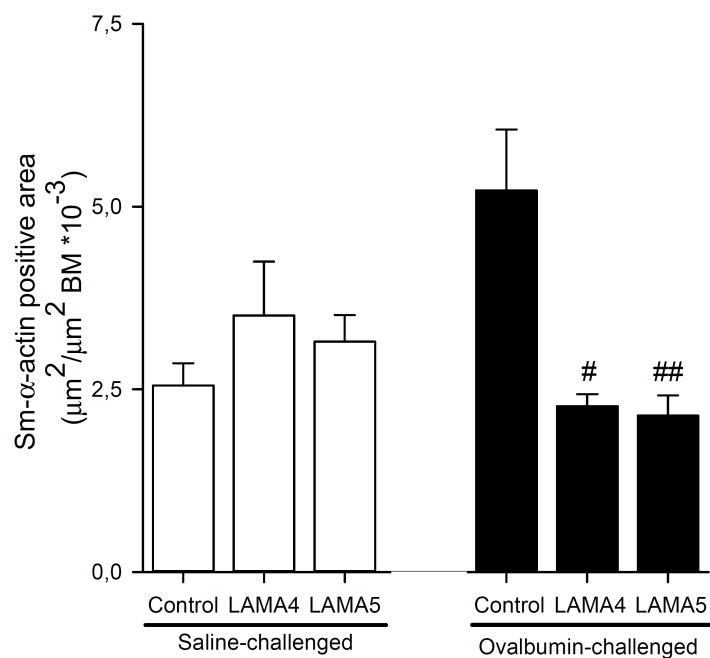


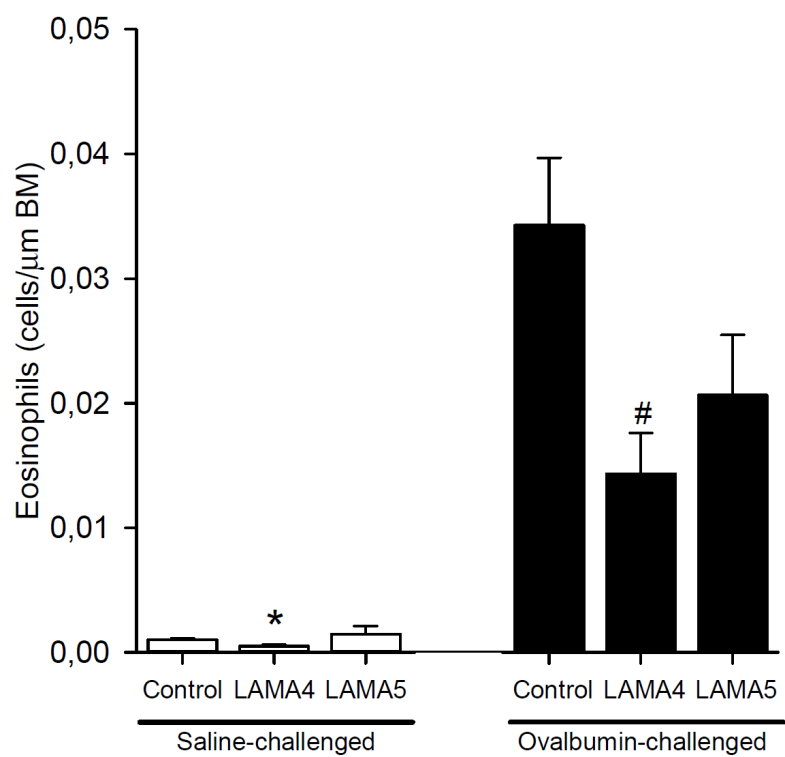
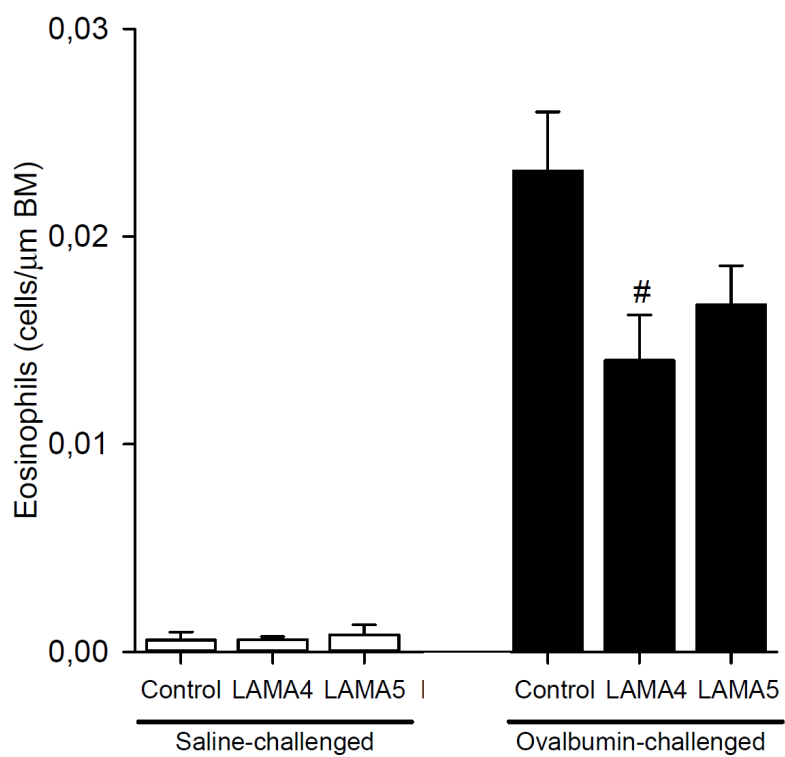
Laminin  $\alpha 4$

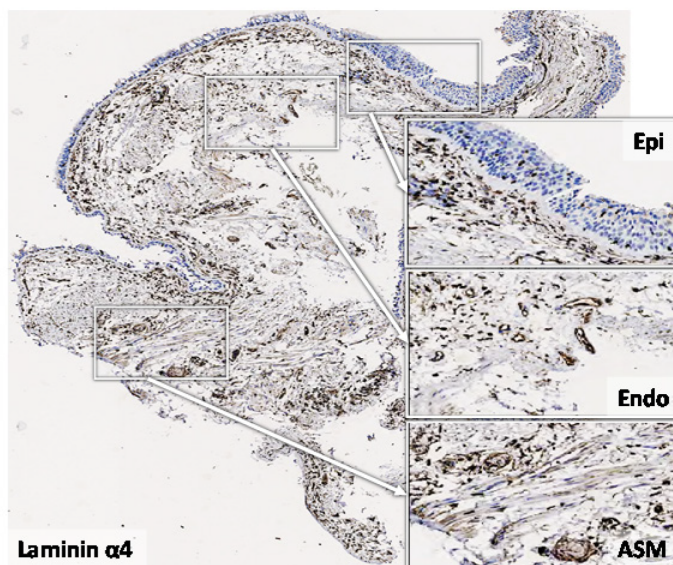
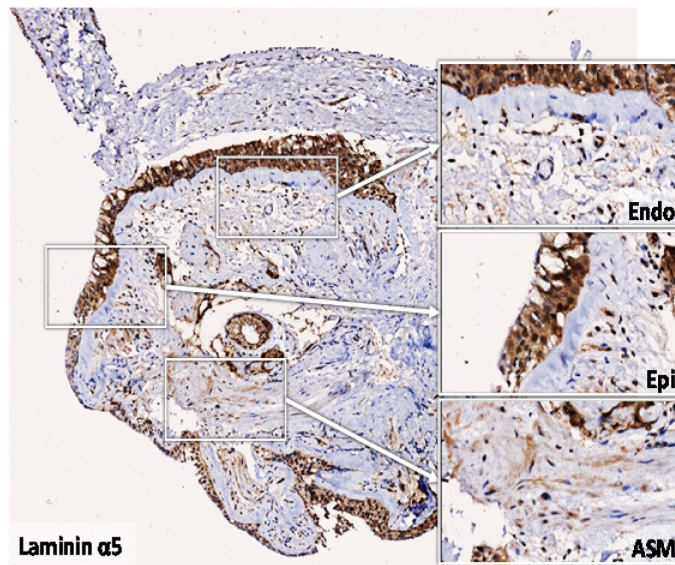
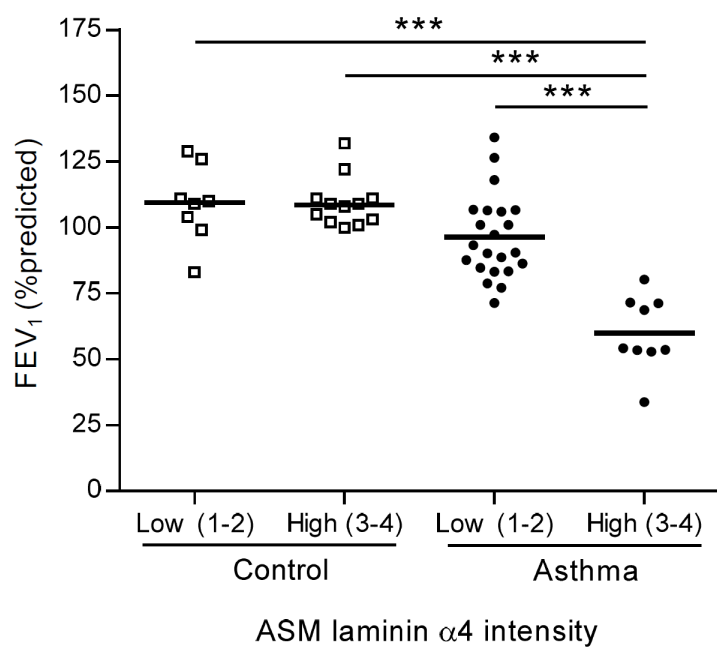
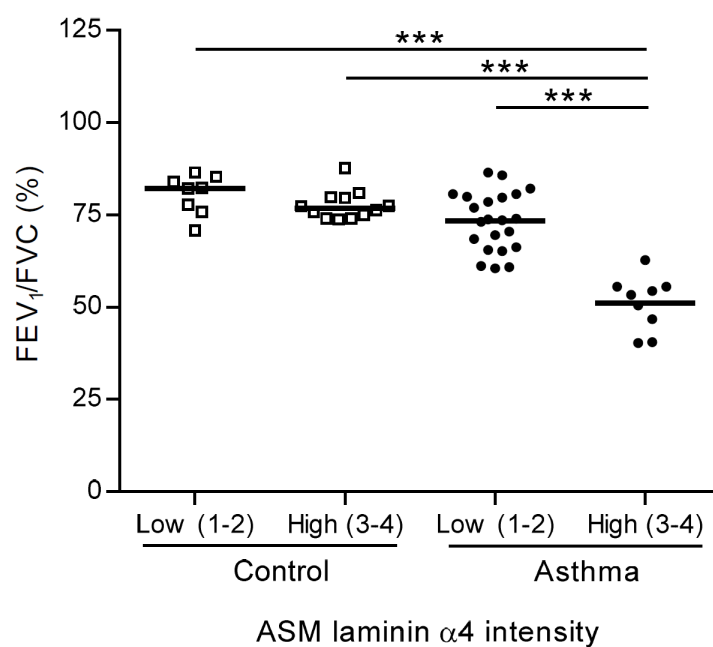
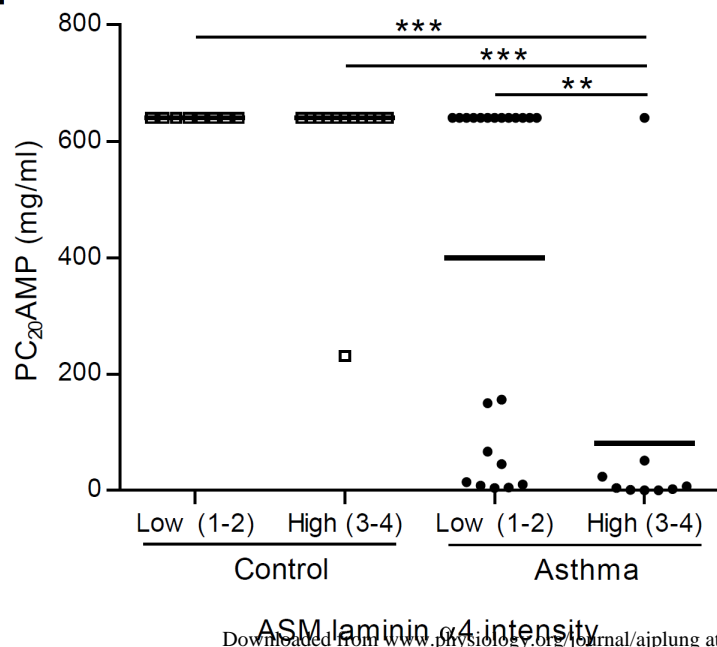
Laminin  $\alpha 5$

**C**

**Chronic Protocol**



**A****B**

**A****B****C****D****E****F**